Morphological and molecular studies on *Garra imberba* and its related species in China

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Abstract: *Garra imberba* is widely distributed in China. At the moment, both *Garra yiliangensis* and *G. hainanensis* are treated as valid species, but they were initially named as a subspecies of *G. pingi*, a junior synonym of *G. imberba*. *Garra alticorpora* and *G. nujiangensis* also have similar morphological characters to *G. imberba,* but the taxonomic statuses and phylogenetic relationships of these species with *G. imberba* remains uncertain. In this study, 128 samples from the Jinshajiang, Red, Nanpanjiang, Lancangjiang, Nujiang Rivers as well as Hainan Island were measured while 1 mitochondrial gene and 1 nuclear intron of 24 samples were sequenced to explore the phylogenetic relationship of these five species. The results showed that *G. hainanensis*, *G. yiliangensis*, *G. alticorpora* and *G. imberba* are the same species with *G. imberba* being the valid species name, while *G. nujiangensis* is a valid species in and of itself.

Keywords: *Garra imberba*; Taxonomy; Morphology; Molecular phylogeny

With 105 valid species *Garra* is one of the most diverse genera of the Labeoninae, and has a widespread distribution ranging from East Asia to Africa (Froese & Pauly, 2012). Menon (1964) first revised the genus and divided it into 4 groups and 9 complexes. *Garra imberbis* and *G. imberba* were classified into the *imberbis* complex, which is distinguished from the other complexes by having more lateral line scales, a larger body size, a shorter distance between vent and the pelvic fin base, and no barbels (Menon, 1964).

Garman (1912) described *Garra* (*Ageneiogarra*) *imberba* using samples from the Min River, Kiating, Szechuan (Leshan, Sichuan), China. *G. imberba* can be distinguished from other *Garra* species by its lack of barbels, 46-52 lateral line scales, 16 circumpeduncle scales, no secondary rostrum, and an anus close to pelvic-fin base. *Ageneiogarra* was treated as a synonym of *Garra*, because the number of barbels was thought to be not eligible to define a new subgenus during the initial intensive study of cyprinid fish. (Karaman, 1971; Kottelat, 1998). Fang (1943) later renamed the materials from Jinshajiang River as *G. pingi*, but Kottelat (1998) treated *G. imberba* as a valid name, and this convention is still widely accepted at present.

Four subspecies of *Garra imberba* have also been identified, although their species validity is still uncertain. Wu et al (1977) described a new subspecies, *G. pingi yiliangensis*, based on the materials from Nanpanjiang River. *G. pingi yiliangensis* has 9 branched dorsal-fin rays, a standard length 5.7−6.1 times body depth, and the caudal-peduncle is 2.0−2.1 times longer than its depth. Zheng & Chen (1983) described another subspecies from Hainan Island, named *G. pingi hainanensis*. It has 46−47 lateral-line scales, and the length of caudal-peduncle is 1.1−1.2 times its depth. *Garra imberba, G. pingi yiliangensis,* and *G. pingi hainanensis* all have isolated

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distributions, so they were treated as distinct species under the phylogenetic species as conceptualized by Kottelat (1998). *Garra pingi* caught from the Nujiang River (upper Salween River) were later described as *G. nujiangensis* by Chen et al (2009). It has 12−14 circumpeduncle scales, and some of its characteristics are meristically and metrically different from *G. imberba*. However, Chen et al also found that the new species has some characteristics similar with *G. imberba*, especially among juveniles, so they just temporarily treated it as a distinct species from *G. imberba*. Lastly, *Garra alticorpora* was named by Chu & Cui (1987) based on two specimens from Pingbian, Yunnan, which belongs to the Red River Drainage system. The most significant characteristic of this *Garra* is that its body depth is longer (vs. shorter) than its head length. After original sampling, however, specimens collected from the same location did not have the same characteristics, so the two *G. alticorpora* samples were thought to be gravid females and treated as a synonym of *G. imberba* by Zhou et al (2005). Besides *G. imberba*, all four of these species (*G. pingi yiliangensis, G. pingi hainanensis, G. nujiangensis, G. alticorpora*) were known as either subspecies of it or considered synonym of it, and their species validity and phyloge netic relationship is still uncertain.

The mitochondrial DNA and nuclear gene have been widely used in the studies of molecular phylogenetic analyses of subfamily Labeoninae or genus *Garra* (He et al, 2008; Li et al, 2005; Tang et al, 2009; Yang & Mayden, 2010; Zheng et al, 2010; Yang et al, 2012). In this study, morphological and molecular methods were used to investigate the phylogenetic relationships among the five species or subspecies of *G. imberba* and its related species distributed in China.

MATERIALS AND METHODS

Sampling

A total of 128 specimens were measured for morphologic analysis, including 90 *Garra imberba* from Jinshajiang and Red-Mekong River, 9 *G. yiliangensis* from Nanpanjiang River, 11 *G. hainanensis* from Hainan Island, 16 *G. nujiangensis* from Nujiang River and 2 *G. alticorpora* from Red River. A total of 24 samples were used in molecular phylogenetic analyses. The collection localities are shown in Figure 1. *Garra yiliangensis* is only distributed in Nanpanjiang and Beipanjiang Rivers(upper Pearl River), but it was not included in this study for molecular analysis because no specimen has been caught in the last decade. No new *G. alticorpora*

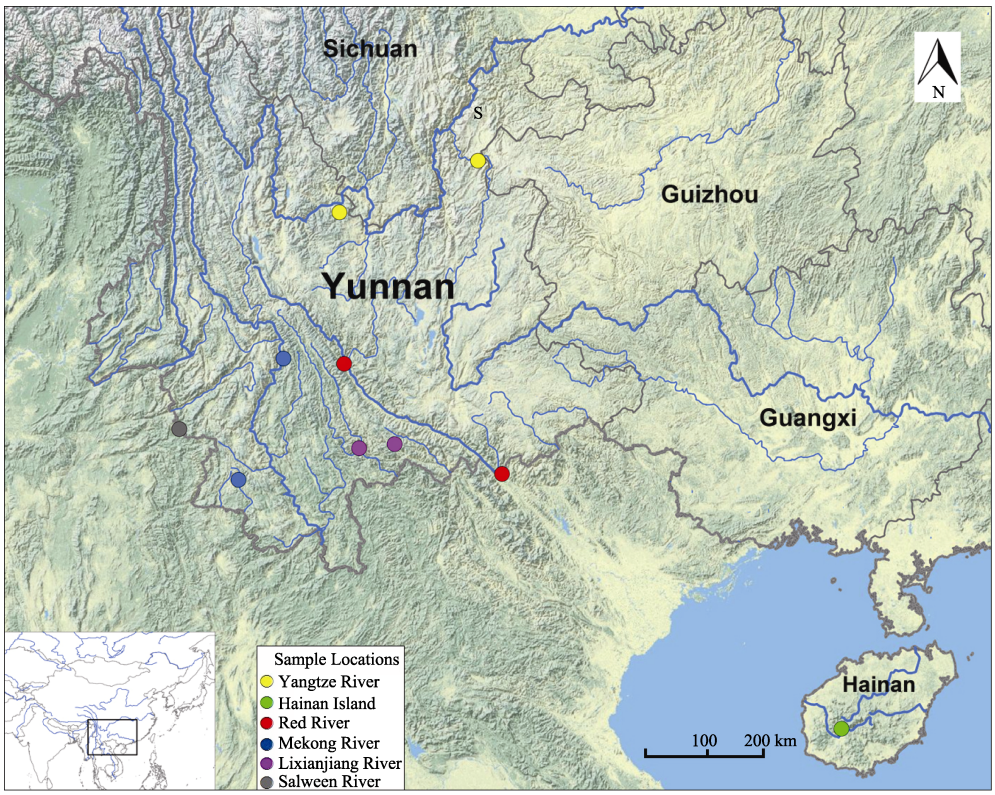

Figure 1 Geographic distribution of samples used in molecular analyses

specimens have been caught since it was named, so it was also not included in the molecular analysis. In addition, one sample of *Garra fasciacauda* from Pu'er, Yunnan was sequenced. This species was thought only to be distributed in the middle of the Mekong River, such as in Thailand and Cambodia, and this is the first record of its presence in China.

All the specimens used in this study are deposited in the Collection Room of Fishes, Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). Voucher information and GenBank accession numbers are listed in Table 1.

Table 1 Taxa used in molecular analyses with source of each samples and GenBank accession numbers for each gene

No.	Drainage	Voucher specimen	Taxon	Location	GenBank Accession No.	
					cyt b	S7
1	YZ1	KIZ041110093	*Garra imberba*	Chuxiong, Yunnan, China	KC119045	KC119071
2	YZ2	KIZ2010001891	*Garra imberba*	Huize, Yunnan, China	KC119046	KC119072
3	RR1	KIZ2010010001	*Garra imberba*	Xinping, Yunnan, China	KC119047	KC119073
4	RR2	KIZ2010010002	*Garra imberba*	Hekou, Yunnan, China	KC119048	KC119074
5	LX1	KIZ2004001193	*Garra imberba*	Puer, Yunnan, China	KC119049	KC119075
6	LX2	KIZ2009002070	*Garra imberba*	Lvchun, Yunnan, China	KC119050	KC119076
7	MK1	KIZ2008000088	*Garra imberba*	Xishuangbanna, Yunnan, China	KC119051	KC119077
8	MK2	KIZ2008008269	*Garra imberba*	Puer, Yunnan, China	KC119052	KC119078
9	HN1	KIZ2008003973	*Garra hainanensis*	Ledong, Hainan, China	KC119053	KC119079
10	HN2	KIZ2008003938	*Garra hainanensis*	Ledong, Hainan, China	KC119054	KC119080
11	SW1	KIZ2005005142	*Garra nujiangensis*	Cangyuan, Yunnan, China	KC119055	KC119081
12	SW2	KIZ2005005145	*Garra nujiangensis*	Cangyuan, Yunnan, China	KC119056	KC119082
13		KIZ2008005732	*Garra micropulvinus*	Wenshan, Yunnan, China	KC119057	KC119083
14		KIZ2011002800	*Garra findolabium*	Jinping, Yunnan, China	JQ864598	KC691274
15		KIZ5171	*Garra cryptonemus*	Liuku, Yunnan, China	JQ864587	KC691275
16		KIZ2007002789	*Garra caudofasciatus*	Jiangcheng, Yunnan, China	JQ864588	KC691276
17		KIZ2004014928	*Garra fasciacauda*	Puer, Yunnan, China	JQ864597	KC691277
18		KIZ2006004460	*Garra tengchongensis*	Tengchong, Yunnan, China	JQ864586	KC691278
19		KIZ2004000816	*Garra dulongensis*	Dulongjiang, Yunnan, China	JQ864590	KC691279
20		KIZ2006004422	*Garra qiaojiensis*	Yingjiang, Yunnan, China	JQ864583	KC691280
21		KIZ2006003543	*Garra salweenica*	Dehong, Yunnan, China	JQ864593	KC691281
22		KIZ2005000086	*Garra orientalis*	Longlin, Guangxi, China	JQ864581	KC691282
23		KIZ2005002392	*Garra mirofrontis*	Yunxian, Yunnan, China	JQ864584	KC691283
24			*Labeo senegalensis*		AB238968	AY103160

Morphological analyses

Measurements were taken point to point with a digital caliper to the nearest 0.1 mm. Measurements and counts were made on the left side of individuals whenever possible (Kottelat, 2001). Predorsal, prepectoral, prepelvic and preanal lengths were taken respectively, from the anterior most tip of the snout to the dorsal-, pectoral-, pelvic- and anal-fin origins. Interorbital width was measured between the upper margins of the eyes. Abbreviations used in this paper are as follows: SL (standard length), HL (head length), HD (head depth), HW (head width), DFL (dorsal fin length), PDL (predorsal fin length), PFL (pectoral fin length), PPL (prepectoral fin length), VFL (ventral fin length), PVL(preventral fin length), AFL (anal fin length), PAL (preanal fin length), CFL (caudal fin length), CPL(caudal

peduncle length), CPD (caudal peduncle depth), DAA (distance between anal and origin of anal fin), DVA (distance between ventral fin base and origin of anal fin), DL (disk length), DW (disk width), ED (eye diameter), IOW (interorbital width), SNL (snout length).

Principal component analysis (PCA) was used to examine the significance of difference among samples of different species or subspecies with SPSS 13.0. Default settings of factor analysis were applied for data standardization. Covariance matrix was used in the analysis. All samples were assigned into 5 groups based on species.

DNA extraction and amplification

Total genomic DNA was extracted from the ethanol preserved specimens using a standard phenol/chloroform isolation and ethanol precipitation. Polymerase chain reaction (PCR) was used to amplify the sequences of one mitochondrial gene and one nuclear gene: cytochrome b (*cyt b*) and the first intron of S7 ribosomal protein (r-protein) gene. These genes are considered to be effective for understanding the evolutionary relationships among populations and species (He et al, 2008; Irwin et al, 1991). The complete *cyt b* gene was amplified with primers L14724 and H15915 (Xiao et al, 2001), S7 was amplified using primers S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998). The PCRs contained approximately 100 ng of template DNA, 1 μL of each primer, 5 μL of 10× reaction buffer, 2 μL dNTPs (each 2.5 mM), and 1 U Taq DNA polymerase in total 50 μL volume. The PCR profile consisted of an initial denaturation step (3 min at 94 ℃), followed by 35 cycles performed in the following order of denaturation at 94 ℃ for 1 min; annealing at 52 ℃ for 1 min; and elongation at 72 ℃ for 1 min; and a final extension at 72℃ for 8 min. PCR amplification products were purified using the Sangon DNA purification kit according to manufacturer's instructions. The purified PCR product was sequenced by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Sequence alignment and phylogenetic analyses

DNA sequences were edited using DNASTAR v7.1.0 (DNASTAR Inc.). All sequences were aligned by Clustal W and checked by eye in MEGA 5.0 (Tamura et al, 2011). Amino-acid translation was also conducted using MEGA 5.0 (Tamura et al, 2011) to confirm the correct reading frame positions and find unexpected stop codons. The genetic distances (p-distance with 1 000 bootstrap) of the two sequences between taxa were calculated using MEGA 5.0 (Tamura et al, 2011). Two operational datasets were constructed for subsequent analyses: (i) mitochondrial DNA sequences, complete cyt-b (1 140 bp); (ii) nuclear DNA sequence, only include the first intron of S7 (805-809 bp).

Phylogenetic analyses were performed using maximum likelihood estimation (ML), and Bayesian inference methods with PAUP* version 4.0 b10 (Swofford, 2003) and MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) respectively. For the ML analyses, a heuristic search was adopted with 1000 bootstrap replications, and a random tree was used as starting tree. Bayesian analyses was conducted for 5 million generations, using one cold and 3 incrementally heated chains and sampling every 100 generations. Two independent runs were performed to confirm convergence. Models were calculated by jModeltest v0.1.1 (Posada, 2008).

RESULTS

Morphological analyses

Counts and proportional measurements of *Garra imberba*, *G. yiliangensis*, *G. hainanensis*, *G. nujiangensis* and *G. alticorpora* are shown in Table 2. The factor analysis results showed that the first 3 components can explain 73.8% of the total variances. For the first 3 components, a scatter graph was drawn as follows (Figure 2). The 5 biggest correlations of the factor analyses of the first 3 components is shown in Table 3. Factor 1 reflects a primarily more slender body shape, and is composed mostly of samples of *G. yiliangensis* that are not clustered with the other studied species. There was no significant difference for factor 2 and 3 among these species.

Molecular analyses

Among the two sequences used in this study, only *cyt b* is a protein coding gene and could be translated into amino acid sequences without interruption. The pairwise distances of *cyt b* and S7 are shown in Tables 4, 5 and 6. The overall mean p distance of clade A1 (*G. imberba* and *G. hainanensis*) based on *cyt b* and S7 are 1.9%±0.3% and 0.2%±0.1%. species of clade A3 contains valid species of *Garra* distributed in China and the mean distance within this clade based on *cyt b* and S7 are 12.3%±0.5% and 11.2%±0.7%. The significant difference within clade A1 and A3 implied that samples of clade A1 actually represent the same species.

Table 2 Counts and proportional measurements of *Garra imberba*, *G. yiliangensis*, *G. hainanensis* and *G. nujiangensis*

	G. imberba				*G. yiliangensis*				*G. hainanensis*				*G. nujiangensis*				*G. alticorpora*			
n	90				9				11				16				2			
Dorsal-fin rays	iv, 8-10				iv, 9-11				iv, 7-8				iv, 8-9				iv, 9			
Pectoral-fin rays	i , 14-15				i , 15-16				i , 14-15				i , 14-15				i , 15			
Pelvic-fin rays	i , 8				i , 8				i , 8				i , 8				i , 8			
Anal-fin rays	ii , 5				ii , 5				ii , 5				ii , 5				ii , 5			
Circumpeduncle scales	16-18				16				16				12-14				16			
Lateral-line scales	47-52				48-51				45-47				47-49				48			
In % of SL	Min	MAX	MEAN	SD	MIN	MAX	MEAN	SD	MIN	MAX	MEAN	SD	MIN	MAX	MEAN	SD	MIN	MAX	MEAN	SD
BD	17.4	24.6	20.5	1.3	17	19.9	17.9	1	17.7	21.7	20.2	1.2	18.5	23.2	21.2	1.5	24.5	26.6	25.6	1.5
HL	19.9	26.5	23.5	1.3	19.4	21	20.1	0.6	21.5	24.4	23.3	0.8	19.8	24	21.9	1.3	20.8	23.4	22.1	1.8
HD	14.4	17.4	15.6	0.7	11.7	14	13	0.7	14.3	16.3	15.4	0.6	13	16.7	14.7	1.1	15.8	17	16.4	0.9
DFL	21.1	31.9	24.4	1.9	20.7	23.6	22.3	0.9	21.8	25.5	23.8	1.1	20.3	24.6	22.8	1.3	23.7	24	23.8	0.2
PDL	44.4	52.4	48.4	1.6	41.9	45.8	43.9	1.2	47.8	51.3	49.8	1.4	45.2	50.4	47.8	1.6	47.8	48.5	48.1	0.5
PFL	17.2	24.5	20.9	1.5	17	20.5	18.8	1.2	17.8	19.9	18.8	0.6	17.5	23.5	20.7	1.6	19.1	19.4	19.3	0.2
PPL	19.6	27.9	22.7	1.4	18.9	20.5	19.8	0.6	20.9	23.7	22.2	0.9	18.8	22.3	20.4	1	20.6	21.2	20.9	0.5
VFL	16.4	23.9	19.3	1.3	16.5	19.6	18.4	0.9	15.9	18.4	17	0.7	17.6	20.5	19	0.9	18.4	19.5	18.9	0.8
PVL	48.6	57.2	52.5	2	45.6	53.3	49	2.4	49.5	54.4	51.7	1.5	50.2	56.3	52.6	1.4	53.3	53.3	53.3	0
AFL	15.7	22.3	18.7	1.4	16.1	18.7	17.4	0.8	15.7	18.6	17.1	0.8	16.4	19.5	17.7	0.8	18.6	18.7	18.7	0.1
PAL	73.5	81.7	77.7	1.8	72.2	80.4	75.9	2.6	75	79.8	77.4	1.6	73.6	79.9	76.3	1.6	77.9	78.1	78	0.1
CFL	25.5	35.8	29.5	2.4	25.9	32.6	29.4	2.3	28.4	31.8	30.1	1	26.4	31.2	28.5	1.4	31.3	32.4	31.8	0.8
CPL	11.9	19.2	15.5	1.5	14.5	20.9	18	2	14.7	16.7	15.8	0.6	15	18.5	16.5	0.9	16.3	16.5	16.4	0.1
CPD	8.7	12.9	10.9	0.7	8.5	10.5	9.2	0.8	9.7	11.1	10.6	0.4	10.1	11.8	10.9	0.4	11.7	13.2	12.5	1
In % of HL																				
HW	66.6	88.2	76.9	4.3	75.7	85.2	79.6	3.2	69.8	79.2	75	2.8	73.7	85.9	78.5	3.2	86.8	89.6	88.2	2
DL	34.8	49.7	40.8	2.8	36.1	45.7	40.3	3.4	35.7	41.6	39.2	1.7	33.9	42.5	38	2	39.2	43.5	41.3	3
DW	45.5	72.3	57.6	5	55.9	67.7	59.6	4.3	47.1	59.9	52.3	3.5	50.7	56.3	52.8	1.6	61.8	53.9	57.8	5.6
ED	18.1	28.7	22.3	2.3	18.4	23.7	20.3	1.7	18.7	21.4	19.6	0.8	20.3	24.4	22.1	1.4	19.7	20.2	19.9	0.3
IOW	47	62.3	53	3.1	51	59.4	55.3	2.7	49.6	55.2	52.6	1.7	50.1	63.5	55.4	3	57	61.2	59.1	2.9
SNL	41.5	56.9	50.5	2.7	53.1	58.3	55.8	1.6	51.4	55.1	53	1.3	47.2	57	52.4	2.6	54.9	57.1	56	1.5
In % of CPL																				
CPD	55.8	93.2	71	7.6	42.9	72.3	51.9	9.8	64.4	74.9	67.3	3.2	57.5	77.2	66.3	5.5	70.9	81	75.9	7.2
In % of DVA																				
DAA	75	86.6	80.9	2.5	79.9	83.8	82.1	1.6	78.8	84.5	82.2	1.7	75.6	82.7	79.2	2	77.9	81	79.4	2.2

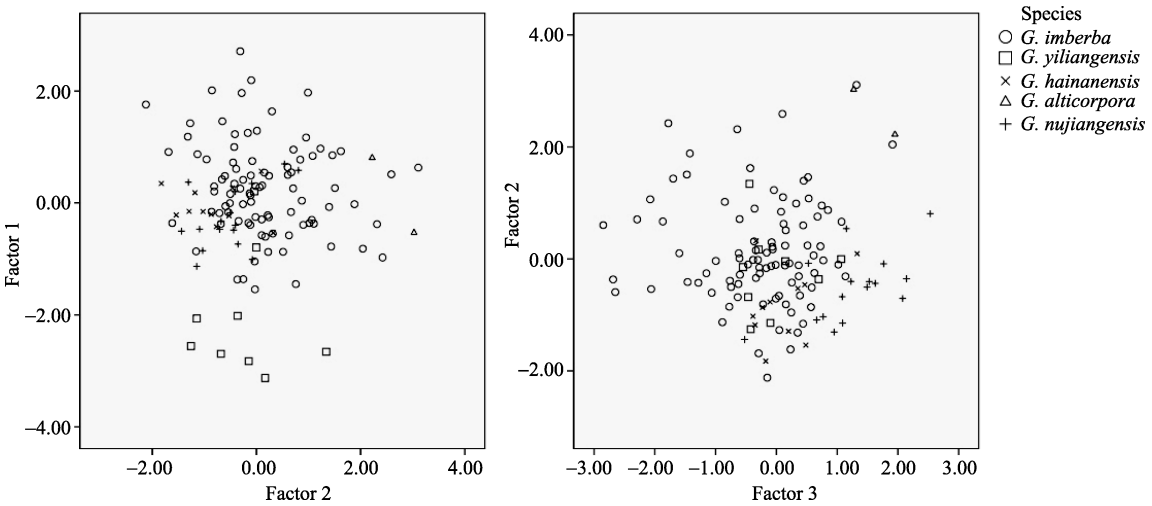

Figure 2 Scatter plots analyzed by PCA for samples from different locality groups based on 22 morphometric characters
A: scatter plot of scores on factor1 and factor 2; B: scatter plot of scores on factor2 and factor3; AL: *G. alticorpora*, from Red River; HN: *G. hainanensis*, from Hainan Island; LX: *G. imberba*, from Lixianjiang, the biggest tributary of Red river; MK: *G. imberba*, from Lancangjiang River; NP: *G. yiliangensis*, from Nanpanjiang River, the upper stream of Pear River; RR: *G. imberba*, from the main stream of Red River; SW: *G. nujiangensis*, from Nujiang River; YZ: *G. imberba*, from Jinshajiang River.

Table 3 Proportion characteristics and principal components extracted from examined materials

No.	Component 1	Correlation	Component 2	Correlation	Component 3	Correlation
1	0.966	CPD/CPL	0.842	DW/HL	0.648	IOW/HL
2	–0.859	CPL/SL	0.773	HW/HL	–0.457	DL/HL
3	0.648	HL/SL	0.577	DL/HL	–0.45	PPL/SL
4	0.606	HD/SL	0.408	IOW/HL	–0.405	HL/SL
5	0.602	PPL/SL	0.352	CPD/SL	–0.404	DAA/DVA

The BI and ML trees of *cyt b* and S7 have the same topology. *Garra imberba* from Jinshajiang, Red, Mekong River and *G. hainanensis* are clustered together, and *Garra nujiangensis* is the sister species of *G. imberba*. In general, phylogenetic relationships based on the phylogenetic trees (Figure 3) of mtDNA exhibit a marked pattern based on geographical distribution, which is different from the result of morphological analyses (Figure 2). *Garra hainanensis* has a closer relationship with *G. imberba* from Jinshajiang and the main stream of Red River, and then clustered with *G. imberba* from Lixianjiang and Lancangjiang River materials. The results show a conflict in the modern river systems; Lixianjiang River is the biggest tributary of Red River, but samples from Lixianjiang have a closer relationship with Lancangjiang River.

G. nujiangensis from Nujiang River can also be easily identified (Figure 4) based on the phylogenetic trees based on the S7, and a mix of samples from Jinshajiang, Red, Lixianjiang, Lancangjiang River and Hainan Island.

DISCUSSION

Morphological discrimination between species

Morphological characteristics are still the most important evidence for defining a new species or subspecies in cyprinid fish. Such analyses usually include two kinds of data: meristic and metric. A difference in meristic characteristics usually can be used as evidence for defining a new species; metric data usually is used to describe a new species or subspecies based on significant difference (Chu & Cui, 1987; Chu & Chen, 1989).

Table 2 shows the details of the morphological characters of these five species. Most of the meristic data do not have significant difference, except for the circumpeduncle scales: *G. imberba* usually has 16 or on rare occasions 18 (1 in 90) circumpeduncle scales; most of *G. nujiangensis* has 12, and only 1 sample has 14 circumpeduncle scales. Number of circumpeduncle scales was treated as very important evidence in defining species, such as *G. qiaojiensis* and *G. orientalis* (12 vs. 16)

Table 4 The pairware *p* distances of *cyt b* computed by MEGA

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Garra imberba YZ1																						
Garra imberba YZ2	0.000																					
Garra imberba RR1	0.001	0.001																				
Garra imberba RR2	0.001	0.001	0.002																			
Garra imberba LX1	0.025	0.025	0.026	0.026																		
Garra imberba LX2	0.028	0.028	0.029	0.029	0.003																	
Garra imberba MK1	0.028	0.028	0.029	0.029	0.017	0.019																
Garra imberba MK2	0.029	0.029	0.030	0.030	0.017	0.019	0.001															
Garra hainanensis CH1	0.008	0.008	0.009	0.009	0.028	0.031	0.032	0.033														
Garra hainanensis CH2	0.008	0.008	0.009	0.009	0.028	0.031	0.032	0.033	0.000													
Garra nujiangensis SW1	0.095	0.095	0.096	0.096	0.091	0.092	0.095	0.094	0.097	0.097												
Garra nujiangensis SW2	0.095	0.095	0.096	0.096	0.091	0.092	0.095	0.094	0.097	0.097	0.000											
Garra micropulvinus	0.104	0.104	0.105	0.105	0.096	0.099	0.103	0.102	0.110	0.110	0.106	0.106										
Garra findolabium	0.111	0.111	0.111	0.111	0.106	0.106	0.106	0.105	0.111	0.111	0.096	0.096	0.116									
Garra cryptonemus	0.128	0.128	0.129	0.129	0.128	0.128	0.130	0.129	0.129	0.129	0.129	0.129	0.128	0.143								
Garra dulongensis	0.139	0.139	0.139	0.139	0.132	0.134	0.132	0.131	0.139	0.139	0.136	0.136	0.141	0.129	0.148							
Garra mirofrontis	0.128	0.128	0.127	0.129	0.121	0.124	0.123	0.122	0.126	0.126	0.127	0.127	0.123	0.125	0.142	0.105						
Garra caudofasciatus	0.123	0.123	0.124	0.124	0.120	0.121	0.127	0.126	0.121	0.121	0.119	0.119	0.118	0.113	0.150	0.138	0.140					
Garra tengchongensis	0.151	0.151	0.152	0.152	0.140	0.141	0.142	0.141	0.148	0.148	0.132	0.132	0.139	0.132	0.143	0.104	0.110	0.142				
Garra orientalis	0.124	0.124	0.125	0.125	0.118	0.120	0.119	0.118	0.125	0.125	0.125	0.125	0.118	0.128	0.139	0.105	0.058	0.143	0.105			
Garra salweenica	0.134	0.134	0.135	0.135	0.129	0.132	0.130	0.129	0.134	0.134	0.132	0.132	0.131	0.127	0.138	0.100	0.059	0.141	0.111	0.063		
Garra qiaojiensis	0.141	0.141	0.140	0.142	0.138	0.139	0.140	0.139	0.143	0.143	0.135	0.135	0.132	0.125	0.144	0.110	0.089	0.136	0.118	0.090	0.096	
Garra fasciacauda	0.126	0.126	0.126	0.127	0.120	0.123	0.125	0.125	0.126	0.126	0.126	0.126	0.130	0.127	0.145	0.131	0.127	0.144	0.130	0.121	0.127	0.132

Table 5 The pairware *p* distances of s7 computed by MEGA

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Garra imberba YZ1																						
Garra imberba YZ2	0.005																					
Garra imberba RR1	0.002	0.006																				
Garra imberba RR2	0.002	0.006	0.000																			
Garra imberba LX1	0.002	0.003	0.003	0.003																		
Garra imberba LX2	0.005	0.000	0.006	0.006	0.003																	
Garra imberba MK1	0.002	0.003	0.003	0.003	0.000	0.003																
Garra imberba MK2	0.002	0.003	0.003	0.003	0.000	0.003	0.000															
Garra hainanensis CH1	0.002	0.003	0.003	0.003	0.000	0.003	0.000	0.000														
Garra hainanensis CH2	0.002	0.003	0.003	0.003	0.000	0.003	0.000	0.000	0.000													
Garra nujiangensis SW1	0.015	0.015	0.014	0.014	0.014	0.015	0.014	0.014	0.014	0.014												
Garra nujiangensis SW2	0.015	0.015	0.014	0.014	0.014	0.015	0.014	0.014	0.014	0.014	0.000											
Garra micropulvinus	0.041	0.043	0.040	0.040	0.040	0.043	0.040	0.040	0.040	0.040	0.040	0.040										
Garra findolabium	0.038	0.040	0.037	0.037	0.037	0.040	0.037	0.037	0.037	0.037	0.037	0.037	0.044									
Garra cryptonemus	0.060	0.061	0.058	0.058	0.058	0.061	0.058	0.058	0.058	0.058	0.060	0.060	0.070	0.064								
Garra dulongensis	0.126	0.126	0.124	0.124	0.124	0.126	0.124	0.124	0.124	0.124	0.121	0.121	0.119	0.124	0.141							
Garra mirofrontis	0.113	0.115	0.112	0.112	0.112	0.115	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.113	0.135	0.072						
Garra caudofasciatus	0.051	0.052	0.049	0.049	0.049	0.052	0.049	0.049	0.049	0.049	0.046	0.046	0.055	0.052	0.070	0.139	0.129					
Garra tengchongensis	0.142	0.142	0.141	0.141	0.141	0.142	0.141	0.141	0.141	0.141	0.138	0.138	0.136	0.139	0.156	0.060	0.080	0.155				
Garra orientalis	0.118	0.119	0.116	0.116	0.116	0.119	0.116	0.116	0.116	0.116	0.116	0.116	0.116	0.118	0.139	0.077	0.014	0.132	0.087			
Garra salweenica	0.110	0.112	0.109	0.109	0.109	0.112	0.109	0.109	0.109	0.109	0.110	0.110	0.109	0.110	0.132	0.072	0.040	0.123	0.080	0.044		
Garra qiaojiensis	0.123	0.124	0.121	0.121	0.121	0.124	0.121	0.121	0.121	0.121	0.121	0.121	0.121	0.123	0.142	0.078	0.052	0.136	0.084	0.060	0.057	
Garra fasciacauda	0.156	0.156	0.155	0.155	0.155	0.156	0.155	0.155	0.155	0.155	0.155	0.155	0.162	0.155	0.172	0.173	0.165	0.164	0.185	0.165	0.176	0.173

Table 6 Overall mean p distance within and between clades computed by MEGA

Sequence	Clade	Mean distance (%)	*SE* (%)
cyt b	A1	1.9	0.3
	A2	0	0
	A3	12.3	0.5
	A1 Vs. A2	9.5	0.8
	A1 Vs. A3	12.7	0.6
	A2 Vs. A3	12.4	0.6
S7	A1	0.2	0.1
	A2	0	0
	A3	11.2	0.7
	A1 Vs. A2	1.4	0.4
	A1 Vs. A3	9.7	0.7
	A2 Vs. A3	9.6	0.7

(Chu & Cui, 1987). *Garra nujiangensis* can be distinguished from all the other species by having 12 circumpeduncle scales (vs. 16) which implies it is a valid species and distinct from *G. imberba*.

The number of branched dorsal fin rays is thought to be a stable characteristic and efficient in defining new species (Chu & Cui, 1987). In this study, we found it is actually not as stable as the circumpeduncle scales. For example, in *G. imberba*, among the 90 examined materials 2 samples have 10 branched dorsal fin rays, 10 have 8, and 72 have 9. The number of lateral line scales usually has a changing range and was treated as a general characteristic in defining species (Chu & Cui, 1987). Meristic data in Table 2 show that these two characteristics have little but not significant difference among *G. imberba*, *G. yiliangensis* and *G. hainanensis*, so it is not reasonable to treat them as distinct species based solely on the above characteristics.

In the PCA analysis, distinction among these five species is not very clear. From Figure 2, it can be seen that *G. yiliangensis* has the most significantly different from the other species, but still has 1 material mixed within it. Component 1 mainly stands for the CPD/CPL, which means most *G. yiliangensis* have a slender body, but the metric character is not reliable in defining species

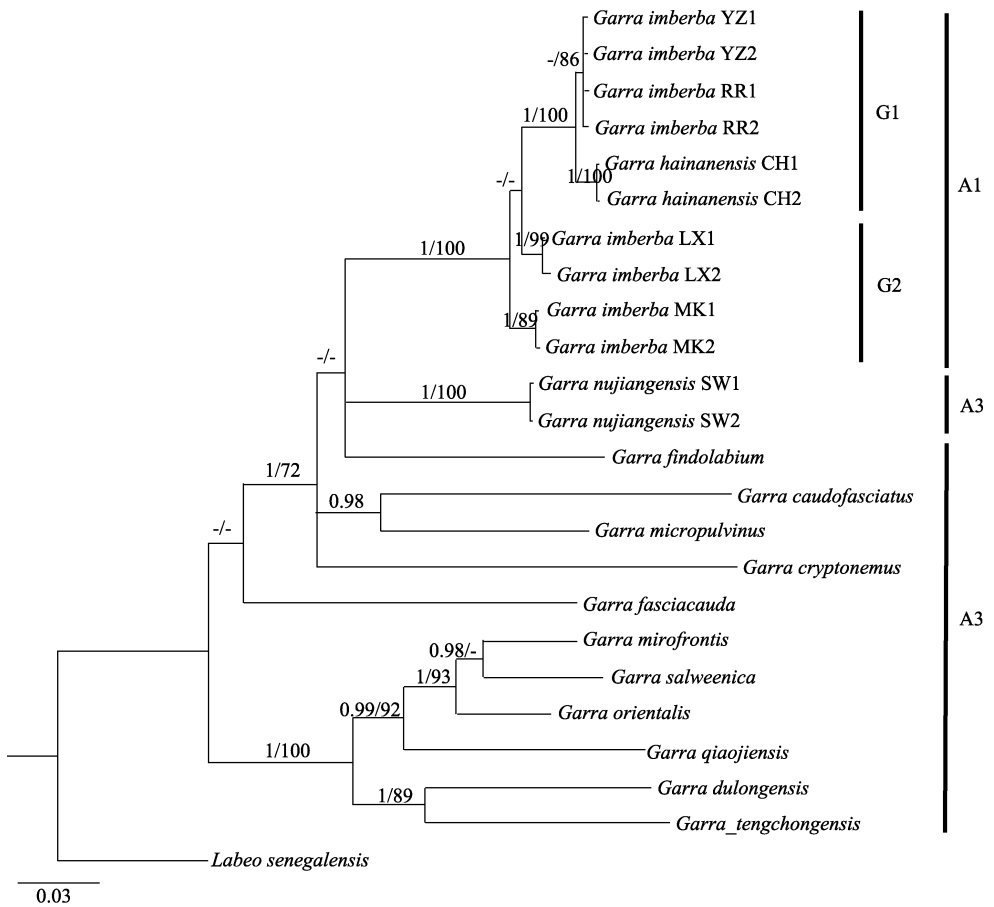

Figure 3 BI tree constructed using MrBayes based on *cyt b* gene

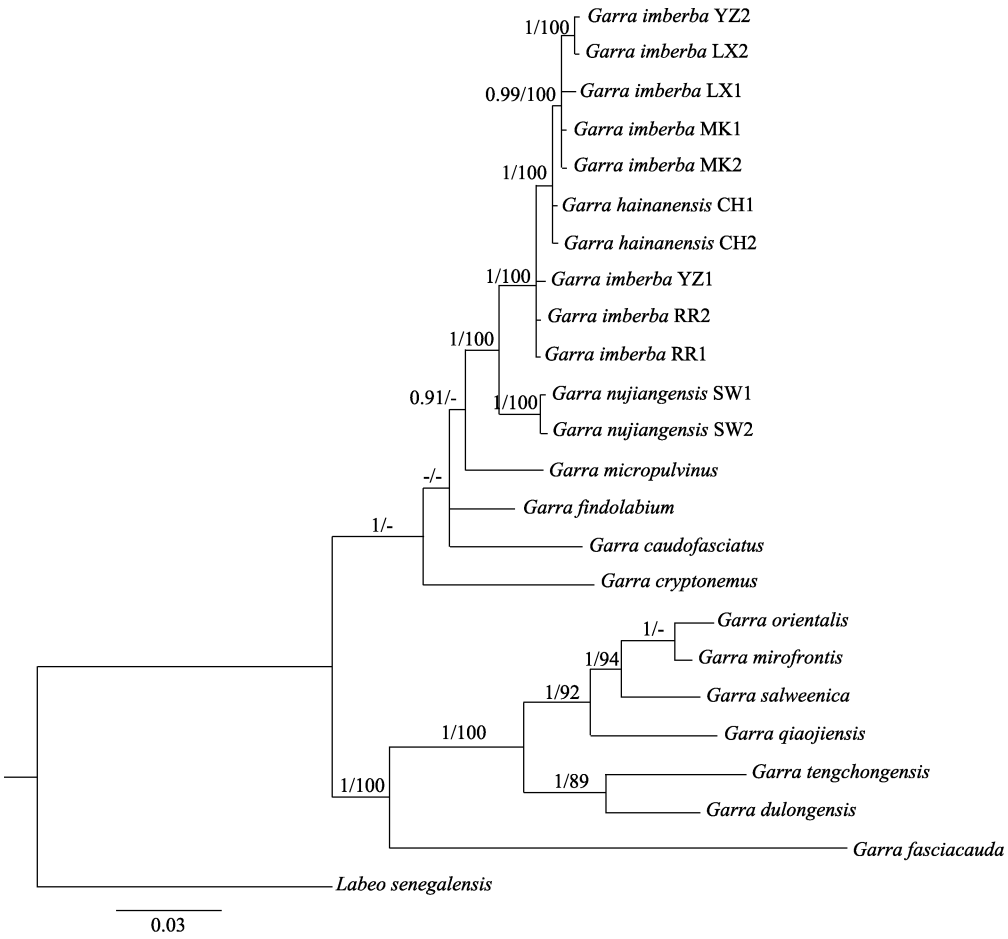

Figure 4 BI tree constructed using MrBayes based on the S7

especially in genus *Garra* (Chu & Cui, 1987). The morphological characteristic is closely related with the environment, for example eyes will degenerate if the fish live in dark caves for a long time. *Garra yiliangensis* has a slender body especially for the caudal peduncle. From an ecological view, it is a characteristic adopted for fast flowing waters. But this difference is not significant, which implies that *G. yiliangensis* from Nanpanjiang River only recently became isolated from adjacent rivers, so it should have a close relationship with *G. imberba* from Jinshajiang and Red River.

Garra alticorpora can only be distinguished from other related species by the fact that its body depth is longer than its head length. There is no difference in meristic characteristics with *G. imberba* and mixed with the others in PCA analysis. The only one holotype and one paratype were collected in Pingbian, Yunnan, which belongs to the Red River Drainage system. In the following surveys, however, only *G. imberba* were caught, so the two specimens of *G. alticorpora* may be just a special case or gravid females and should be treated as a synonym of *G. imberba* (Zhou et al, 2005).

From traditional morphological characteristics analyses, only *G. nujiangensis* can be distinguished from other species. *Garra imberba*, *G. yiliangensis*, *G. hainanensis* and *G. alticorpora* are relatively indifferent from one another, and should be assigned to the same species. Because of the geographical isolation, *G. yiliangensis* and *G. hainanensis* should be treated as different geographical populations of *G. imberba*.

Molecular phylogenetic relationship

It is widely accepted that Jinshajiang, Nanpanjiang and Red River have a close relationship; indeed, Lancangjiang, Nujiang and Irrawaddy River have a close relationship (Chu, 1986). Based on geological studies, sea-level changes during the Pleistocene may have reached the maximum lows, which means the

Red River might have been linked together with rivers on Hainan Island (Hanebuth et al, 2000; Voris, 2000). Therefore, *G. hainanensis* and *G. imberba* from Red and Jinshajiang River possibly have the same ancestor, and split only after the Pleistocene era, which may explain why *G. hainanensis* has a closer relationship with *G. imberba* from Red and Jinshajiang River, and why they were clustered in a lineage of G1. The close relationship coincides with the geographical process of the three rivers.

The Mekong River has the highest fish diversity in Southeast Asia and its fish fauna is also different from the Red, Jinshajiang and Nanpanjiang Rivers (Froese & Pauly, 2012; Yap, 2002; Zakaria-Ismail, 1994). Lixianjiang (Song Da in Vietnam) is the biggest tributary of the Red River, which originated from the Wuliang Mountain, Yunnan, China and flows into the Red River at Viet Tri, Vietnam, but the mtDNA analysis shows that, specimens from Lixianjiang River have a closer relationship with Lancangjiang than the Red River. Zhou et al (2010) compared the morphometrics of Vanmanenia tetraloba from Lancangjiang, Lixianjiang and main stream of Red River. Their results show that specimens from different rivers actually represented different species. *Garra imberba* is not a fish that migrates long distances, and so a gene exchange between the main stream of Red and Lixianjiang would be very difficult given the distance. Therefore, *G. imberba* from Lixianjiang and the main stream of the Red River would not have exchanged genes for a long time, and they were not clustered in the same clade. Clade A1 can be divided into two lineages, G1 and G2, which coincide with the geographical process and the fish fauna of the rivers but conflict with the morphological classification of *G. imberba* and related species. Furthermore, the mean p-distance within the clade A1 is 1.9%, and the biggest p-distance within clade A1 is 3.2%, which is between *G. hainanensis* and *G. imberba* from Lancangjiang River. This value is smaller than the minimum value within the genus *Garra* (Yang et al, 2012). With respect to the evolutionary history and phylogenetic relationship of the samples from different rivers, it is better to treat them as the same species.

The 1st intro of S7 ribosomal protein gene was thought to be an efficient marker for the phylogenetic analyses of subfamily level (He et al, 2008). It has a faster evolution rate than the coding nuclear gene but a slower rate than mtDNA, so we used it here to explore the relationship at a species level. Based on the NJ tree of S7 data, *G. nujiangensis* from Nujiang River and *G. micropulvinus* can be easily identified (Figure 4), which implies *G. nujiangensis* is a valid species and S7 gene is an efficient means for exploring phylogenetic relationships within genus *Garra*. *Garra imberba* and *G. hainanensis* were mixed together and the mean *p*-distance within this clade is 0.2%. It is obvious that *G. imberba* and *G. hainanensis* are the same species and distinct from *G. nujiangensis*.

CONCLUSION

For the morphological analysis, a stable meristic characteristic can be treated as an important proof when defining a new species, such as circumpeduncle scales, but metric characteristics should be considered carefully, especially in exploring relationships between species. Molecular phylogenetic analysis is an efficient method in estimating the phylogenetic relationships between different species or geographical groups. To make a reasonable taxonomy, the morphological differences, phylogenetic relationship and the biogeographic processes should all be considered.

In this study, both morphologic and molecular analyses testified that *G. nujiangensis* is a valid species and distinct from *G. imberba*. Samples from Jinshajiang, Red, Lixianjiang, Nanpanjiang, Lancangjiang River and Hainan Island have the same ancestor, which was isolated because of the formation of modern river systems. Different environments usually lead to different morphological characteristics, but the differences between these materials is not enough to define new species. Based on the morphological and molecular analyses, the samples from Jinshajiang, Nanpanjiang, Red, Lancangjiang River and Hainan Island have a close phylogenetic relationship and should be treated as the same species, and because of the geographical isolation, *G. yiliangensis* and *G. hainanensis* should be treated as different geographical populations of *G. imberba*.

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Materials examined

Garra imberba

Lancangjiang River: KIZ200800826-829, 3, 97.8−182.1mm SL, Zhaji River, Jingfu Xiang, Jingdong, Yunnan; KIZ2002008504-8512, 9, 60.5-103.3 mm SL, Mengga River, Jinggu, Yunnan; KIZ2008000087-89, 2, 83.1−128.6 mm SL, Menggen county, Lancang, Yunnan; KIZ2008008269, 1, 97.6 mm SL, Puer, Yunnan.

Main stream of Red River: KIZ1964000024-25, 2, 14.7−16.2 mm SL, Yuanjiang, Yunnan; KIZ2007002528, 1, 19.4mm SL, Hekou, Yunnan; KIZ2008000922, KIZ2008000927, KIZ2008000929, KIZ2008000932, KIZ2008000936, KIZ2008000938-940, 8, 15.1−20.7 mm SL, Namoguo, Wenshan, Yunnan; KIZ2010010001, 1, 102.9 mm SL, Xinping, Yunnan; KIZ2010010002, 1, 71.6 mm SL, Simao, Yunnan.

Jinshajiang River: KIZ1982001210-1224, 15, 47.4−118.85 mm SL, Yanjin, Yunnan; KIZ2004011252-253, 2, 69.8−195.0 mm SL, Yongsheng, Lijiang, Yunnan; KIZ2008006836, KIZ2010001891, 2, 101.5−136.33 mm SL, Huize, Yunnan; KIZ041110093-99, 6, 91.2−150.7 mm SL, Yongren, Yunnan; KIZ1977000732-734, KIZ1977-000737-738, 5, 51.1−73.1 mm SL, Heqing, Yunnan; KIZ2008006837-40, 4, 108.5−159.3 mm SL, Huize, Yunnan.

Lixianjiang River: KIZ2000001439-1454, 16, 64.5−163.1 mm SL, Jinping, Yunnan; KIZ2007002903-2906, 4, 68.3−107.2 mm SL, Jiangcheng, Yunnan; KIZ2008002405-2416, 12, 102.0−130.6 mm SL, Simao, Yunnan; KIZ2004001193, 1, 82.0 mm SL, Puer, Yunnan; KIZ2009002070, 1, 53.8 mm SL, Lvchun, Yunnan.

Garra yiliangensis

KIZ1960000569, KIZ1963000385, KIZ197700093-3-934, 4, 146.1−187.0 mm SL, Yiliang, Yunnan; KIZ1977000954-958, 5, 166.3−252.5 mm SL, Luoping, Yunnan.

Garra hainanensis

KIZ2008003938, KIZ2008003947, KIZ2008003967, KIZ2008003973-3974, KIZ2008003979, KIZ2008003990, KIZ2008003995, KIZ2008004006, KIZ2008004018, KIZ2008004030, KIZ2008004039, 12, 93.6−117.7 mm SL, Ledong, Hainan.

Garra nujiangensis

KIZ2003007967 (Holotype), KIZ2003000279 (Paratype), KIZ2003000272, KIZ2003000278, KIZ2003000281-282, KIZ2003000288-289, KIZ20030-00291, KIZ2003000294, 10, 82.5−186.7 mm SL, Zhenkang, Yunnan; KIZ2005005140-145, 6, 117.8−173.0 mm SL, Cangyuan, Yunnan;

Garra alticorpora

KIZ1985001344 (Holotype), KIZ1985001345 (Paratype), 2, 164.6−165.6 mm SL, Pingbian, Yunnan.

Garra micropulvinus

KIZ2008005732, 1, 83.4 mm SL, Wenshan, Yunnan.

Garra findolabium

KIZ2011002800, 1, 36.9 mm SL, Jinping, Yunnan.

Garra cryptonemus

KIZ5171, 1, 13.5 mm SL, Liuku, Yunnan.

Garra caudofasciatus

KIZ2007002789, 1, 76.1 mm SL, Jiangcheng, Yunnan.

Garra fasciacauda

KIZ2004014928, 1, 56.3 mm SL, Puer, Yunnan.

Garra tengchongensis

KIZ2006004460, 1, 53.3 mm SL, Tengchong, Yunnan.

Garra dulongensis

KIZ2004000816, 1, 98.5 mm SL, Dulongjiang, Yunnan.

Garra qiaojiensis

KIZ2006004422, 1, 112.1 mm SL, Yingjiang, Yunnan.

Garra salweenica

KIZ2006003543, 1, 74.6 mm SL, Dehong, Yunnan.

Garra orientalis

KIZ2005000086, 1, 138.9 mm SL, Longlin, Yunnan.

Garra mirofrontis

KIZ2005002392, 1, 88.6 mm SL, Yunxian, Yunnan.

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